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Ecological considerations in producing and formulating fungal entomopathogens for use in insect biocontrol

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Abstract Insect pests persist in a wide-variety of agricultural, arboreal and urban environments. Effective control with fungal entomopathogens using inundation biocontrol requires an understanding of the ecology of the target insect, fungal pathogen, and the insect-pathogen interaction. Historically, the development of production and formulation processes for biocontrol fungi has primarily focused on reducing costs by maximizing the yield of infective propagules, increasing storage stability, and improving product form for ease of application. These goals are critical for commercialization but are often in conflict with environmental and ecological considerations. Critical parameters for selecting a fungal pathogen for use in inundation biocontrol include the cost-effective production of a stable, infective propagule that is suited for use in the environment where the insect must be controlled. Production processes can be manipulated nutritionally and environmentally to produce efficacious propagules or to direct fungal differentiation to propagule forms that may be better suited for use in specific environments. Formulation development must also consider ecological and environmental factors to maximize biocontrol efficacy. A basic understanding of the surface chemistries of the fungal propagule and insect, the interactions between a fungal propagule and the insect cuticle that lead to infection, and the impact of the environment on this interaction can aid in the development of effective formulations.

Keywords Biocontrol · Fungi · Fermentation · Formulation · Conidia · Blastospores · Sclerotia · Mycoinsecticides

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Introduction

Over the past 50 years, the control of insects, weeds, and plant diseases with fungal pathogens has been a very active area of research and has resulted in a large number of commercially-available products (Butt et al. 2001; Charudattan 2001; Wraight et al. 2001; Fravel 2005; Faria and Wraight 2007). The commercial use of fungal entomopathogens to control insects is generally practiced using the inundation biocontrol approach where the environment harbouring the insect pest is inundated with high concentrations of infective fungal propagules (Eilenberg et al. 2001).

Products developed for use in the inundative approach are often termed "mycoinsecticides" or "biopesticides" in reference to their similar usage pattern compared to chemical insecticides.

Fungi are unique candidates for use in "inundation" biocontrol because of their ability to actively infect and kill the target pest. The high number of fungal propagules used in this approach requires a cost-effective production and stabilization process that delivers viable, infective fungal propagules (Goettel and Roberts 1992; Wraight et al. 2001; Jackson 2007). Production and formulation are critical to the commercial development of a fungal biocontrol agent. The biocontrol agent must provide a cost—benefit to the end user (be low cost), have a reasonably long shelf-life (remain viable and infective during storage), and provide consistent insect control under field conditions (function well in the environment of use). Shortcomings in any of these qualities can prevent the agent from becoming a commercial product. In general, product cost and storage stability have driven the development of production and formulation processes. Often, these production goals are in conflict with ecological and environmental requirements for consistent infectivity and control of the insect target. A more thorough understanding of the relationship between the insect pest and the agricultural system being managed is needed to assure success in using mycoinsecticides as stand-alone products or as part of an integrated approach to pest management (Thomas 1999; Shah and Pell 2003; Lacey and Shapiro-Ilan 2008). Many of the papers in this special issue deal with specific issues related to these "insect–pathogen–environment" interactions.

More than 150 insect biocontrol products based on fungal entomopathogens have been commercialized with over 75% of these products based on the hypocrealean fungi *Metarhizium anisopliae*, *Beauveria bassiana*, *Isaria fumosorosea*, and *B. brongniartii* (Faria and Wraight 2007). Two-thirds of these commercialized products are comprised of conidial preparations of *B. bassiana* or *M. anisopliae*, presumably using solid-substrate fermentation production processes. Both *B. bassiana* and *M. anisopliae* have a very broad insect host range with many isolates producing high concentrations of aerial conidia when grown on nutrient-rich, solid substrates (Jaronski 1997). The use of conidia as mycoinsecticides is warranted as they are a naturally-infective propagule. There are, however, ecological and environmental conditions in which the use of conidia may not be the best choice for insect biocontrol in agricultural or urban settings. For example, conidia are more shelf stable at room temperature when compared to blastospore preparations of *I. fumosorosea* but, following rehydration, can take up to 24 h to germinate compared to a 6 h germination time for rehydrated blastospores (Vega et al. 1999). Studies with subterranean termites have shown that fungal conidia readily attach to the cuticle but are effectively removed by mutual grooming (Yanagawa et al. 2008). The delivery of a blastospore preparation or other fungal propagule that germinates more rapidly may be a more effective propagule choice for control of social insects that groom nest mates or for insects that frequently moult.

Production and formulation strategies for potential mycoinsecticides must consider the environmental and ecological requirements and limitations (Vega et al. 2009). From a biotechnology standpoint, a variety of fungal propagules can be produced using solid-substrate and deep-tank fermentation by altering nutritional and environmental conditions. Likewise, formulations can be employed that alter the chemical and physical attributes of a fungal propagule for improved insecticidal activity under varied environmental conditions. Formulations developed with living, fungal entomopathogens for use in inundation biocontrol must take into account the environmental and ecological life histories of the target insect while maintaining propagule viability and efficacy (Jaronski 1997). This chapter will describe production and formulation strategies that focus not only on economic factors but also on developing fungal propagules designed for insect control in specific environments.

Selecting fungal propagules for use in inundation biocontrol

The identification of the appropriate fungal pathogen for development as a mycoinsecticide can be complex. The selection process must evaluate the potential of the fungal isolate to form a stable propagule that can be economically mass-produced, that is amenable to available application technologies, and, most

importantly, is capable of consistently infecting and killing the target insect under the environmental and ecological conditions where it is a pest (Wraight et al. 2001; Jackson and Schisler 2002; Jaronski 2007). Few fungal entomopathogens are capable of meeting all these requirements. The environmental conditions present during insect control must be considered and appropriate fungi and fungal propagules selected for use in inundation biocontrol. Critical environmental factors, such as temperature, can have a profound influence on the growth and pathogenicity of a fungal entomopathogen against the target insect (Inglis et al. 1996; Faria and Wraight 2001; Yeo et al. 2003). For example, the conidia of fungal isolates collected from environments differing in climatic conditions showed dramatic variation in temperature tolerance. At 48°C, the LT_{50} was 14.3–150.3 min for conidia of various isolates of *Metarhizium* species, 10.1–61.9 min for *B. bassiana* isolates, and 2.8–6.2 min for isolates of *I. fumosorosea* (Li and Feng 2009). This variation in thermal tolerance would be a significant factor in selecting an appropriate entomopathogen for development as a mycoinsecticide

Insects inhabit diverse environments and are pest problems in agricultural, urban, forest, freshwater, and natural ecosystems. Their life histories coupled with environmental conditions conspire to make consistent insect control under field conditions difficult to achieve using fungal entomopathogens. A key consideration in the selection of a fungal entomopathogen is the fungus' ability to produce a suitable propagule for control of the insect. The efficacy of a fungal propagule is dependent on the requirements for use as a mycoinsecticide and may include enhanced virulence, desiccation tolerance, thermal tolerance, speed of germination and infection, environmental stability and reproduction, and UV tolerance (Jackson and Schisler 1992; Jackson et al. 1997; Vega et al. 1999). Numerous studies have shown that nutritional and environmental conditions during fungal growth using solid-substrate and liquid-culture fermentation influence the form and efficacy of the fungal propagule (Hallsworth and Magan 1994, 1995, 1996; Jackson et al. 1996; Jackson 1997; Magan 2001; Ying and Feng 2006). Formulation of the fungal propagule or the use of adjuvants during application can also influence efficacy (Jaronski 1997; Costa et al. 2008; Friesen et al. 2006). The selection of a fungal entomopathogen that economically produces a stable propagule which provides consistent insect control under field conditions is the ultimate goal of the selection process. An excellent description of the requirements for germination, infection and reproduction by fungal entomopathogens on the insect cuticle has been presented by Boucias and Pendland (1991) and Castrillo et al. (2005).

The life history of the insect pest and the environment in which it will be controlled dictate the fungal propagule needed for use as a mycoinsecticide. If the mycoinsecticide is to be applied as a spray (i.e., "contact" biopesticide), the production method must yield high numbers of discrete, infective propagules. Granular mycoinsecticide formulations for use in soil require the production of a persistent fungal propagule that is capable of delivering an infective inoculum to the insect host when required. Many spore forms used in spray applications are not amenable to use in granular applications. Recently, it was shown that some isolates of the entomopathogenic fungus *M. anisopliae* differentiated to form sclerotial propagules when grown in liquid culture fermentation (Jackson and Jaronski 2009). These sclerotial propagules were desiccation tolerant and germinated sporogenically in soil to produce conidia in situ that infected and killed susceptible soil-dwelling insects. The sclerotia-containing granules were more efficacious when compared to granules made from conidia of *M. anisopliae* bound to a solid nutritive carrier (Jaronski and Jackson 2008).

Production of fungal propagules for use in inundation biocontrol

Conidia production using solid substrate fermentation

Inundation biocontrol for foliar insect pests is generally practiced by spraying high concentrations of infective fungal spores. Because they can be produced in high concentration, either aerial conidia or "yeast-like" blastospores are the fungal spore forms commercially-produced for use in the spray application of mycoinsecticides. Application rates for insect control using fungal entomopathogens can approach $2.5–5 \times 10^{13}$ spores ha^{-1} in inundation biocontrol (Faria and Wraight 2001), although lower rates have

been observed to be efficacious, e.g., 2.5×10^{12} conidia ha^{-1} in the case of *M. anisopliae var. acridum* against African Orthoptera (van der Valk 2007).

The primary infective form of most fungal entomopathogens is the conidium and, in fact, the solid substrate production of aerial conidia is the most widely used production method for the mycoinsecticides *Metarhizium* and *Beauveria* (Bartlett and Jaronski 1988; Faria and Wraight 2007). Solid substrate production processes for aerial conidia can be very simple but labour intensive (autoclaved bags of moistened grain inoculated with an entomopathogen) or involve a more automated tray production system requiring higher capital costs with reduced manpower requirements (Bartlett and Jaronski 1988). Other fungi are not suited for solid substrate conidia production. Isolates of the fungal entomopathogen *I. fumosorosea* require light for significant conidia production, a characteristic that has limited its production using solid substrate fermentation (Sanchez-Murillo et al. 2004; Zimmermann 2008). Fortunately, *I. fumosorosea* and other fungal entomopathogens are dimorphic fungi and are capable of growing "yeast-like" in liquid culture to produce blastospores, which can be utilized in spray application after proper drying and formulation (Jackson 1999; Kassa et al. 2004; Jackson et al. 2006).

Submerged conidia production

Both *B. bassiana* and *M. anisopliae var acridum*, but not *M. anisopliae*, will produce submerged or microcycle conidia under certain liquid fermentation conditions (Thomas et al. 1986; Jenkins and Prior 1993; Kassa et al. 2004). These submerged conidia are not hydrophobic, unlike aerial conidia, and thus present different challenges in formulation and use. The microcycle conidia of *B. bassiana* are produced after 96 h of fermentation only in the presence of inorganic nitrogen, as nitrate, and with very high levels of carbohydrate. Submerged conidia are morphologically different from aerial conidia on an ultrastructural level, lacking one layer to their cell walls (Hegedus et al. 1990). Germination speed for submerged conidia is intermediate between aerial conidia and blastospores. Submerged conidia of *M. anisopliae var acridum* were produced on structures very similar to aerial phialides and were morphologically indistinguishable from aerial conidia, although they possessed different physical properties (Leland et al. 2005). Nitrogen, in the form of brewer's yeast, in the presence of excess sucrose was found to be essential for the production of submerged conidia by *M. anisopliae var acridum* cultures. At the present time, submerged conidia have not been commercially developed as an insect biocontrol propagule.

Blastospore production using liquid culture fermentation

Blastospores are vegetative fungal propagules that are the preferred mode of growth for many entomopathogens in the haemocoel of infected insects (Shimuzu et al. 1993; Sieglaff et al. 1997; Vestergaard et al. 1999; Askary et al. 1999). Yeast-like growth allows the fungus better access to the nutrients within the insect. Numerous entomopathogens of the genera *Isaria*, *Beauveria*, *Lecanicillium*, and *Metarhizium* can be induced to grow in a "yeast-like" fashion in submerged liquid culture. Blastospore-based mycoinsecticides are currently produced commercially by *L. lecanii* (Ascomycota: Hypocreales) and *I. fumosorosea* (Faria and Wraight 2007). Our studies with the fungal mycoinsecticide *I. fumosorosea* have demonstrated that desiccation tolerant blastospores can be rapidly produced in high concentrations if an appropriate source and concentration of nitrogen are provided (Jackson et al. 2003). Blastospores of *I. fumosorosea* are highly infective against a number of insect pests and often have a lower LD_{50} when compared to conidial preparations (Poprawski and Jackson 1999; Behle et al. 2006; Shapiro-Ilan et al. 2008).

The rapid germination rate of *I. fumosorosea* blastospores (>90% germination in 6 h) make these propagules ideal candidates for use as a contact mycoinsecticide (Vega et al. 1999). Considering environmental and ecological factors, the rapid germination rate of blastospores reduces the time required for available free-moisture and mitigates the adverse effects of extended exposure in the environment. Furthermore, the rapid germination rate of blastospores increases their chance of infecting moulting insects or social insects that groom nest mates. Blastospores of *I. fumosorosea* have also been shown to be less repellent to the Formosan subterranean termite, *Coptotermes formosanus*, when

compared to conidial preparations of *I. fumosorosea* produced on solid-substrate, rice cultures (Wright et al. 2003). These differences suggest that the properties of aerial conidia, submerged conidia, and blastospores can be exploited for improved insect biocontrol, particularly if the insect target is susceptible to multiple entomopathogens capable of forming these propagules using commercial production methods. The insect's life histories and environment will dictate the appropriate fungal propagule for use as an inundative biocontrol agent.

Sclerotia production using liquid culture fermentation

Sclerotia are compact hyphal aggregates that often become melanized as they develop (Coley-Smith and Cooke 1971). These fungal structures have been reported as the overwintering propagule for many plant pathogenic fungi and for a limited number of fungal entomopathogens (Speare 1920; Evans and Samson 1982). Like many plant pathogenic fungi, sclerotial bodies of the fungal entomopathogen *Nomuraea rileyi* (Ascomycota: Hypocreales) found in insect cadavers were shown to produce infective conidia *via* sporogenic germination in the following growing season (Sprenkel and Brooks 1977; Speare 1920). Recently, it was shown that the fungal entomopathogen *M. anisopliae* produced high concentrations of microsclerotia (small sclerotia) under specific nutritional conditions during liquid culture fermentation (Fig. 1; Jackson and Jaronski 2009). These microsclerotia were desiccation tolerant with excellent storage stability following air-drying. When air-dried microsclerotial granules of *M. anisopliae* were soil-incorporated, they produced infective conidia *via* sporogenic germination following rehydration (Fig. 1; Jaronski and Jackson 2008). During the production of sclerotia in liquid culture, melanin biosynthesis can be controlled with nutrition or culture age (Jackson and Schisler 1995; Shearer and Jackson 2006; Jackson and Jaronski 2009). Fungal melanins have been shown to have allelopathic and antimicrobial properties, act as anti-desiccants, enhance cell rigidity, and confer fungicide resistance, all properties that would enhance the vigour of sclerotial propagules for use as a mycoinsecticide in the rhizosphere (Butler and Day 1998).

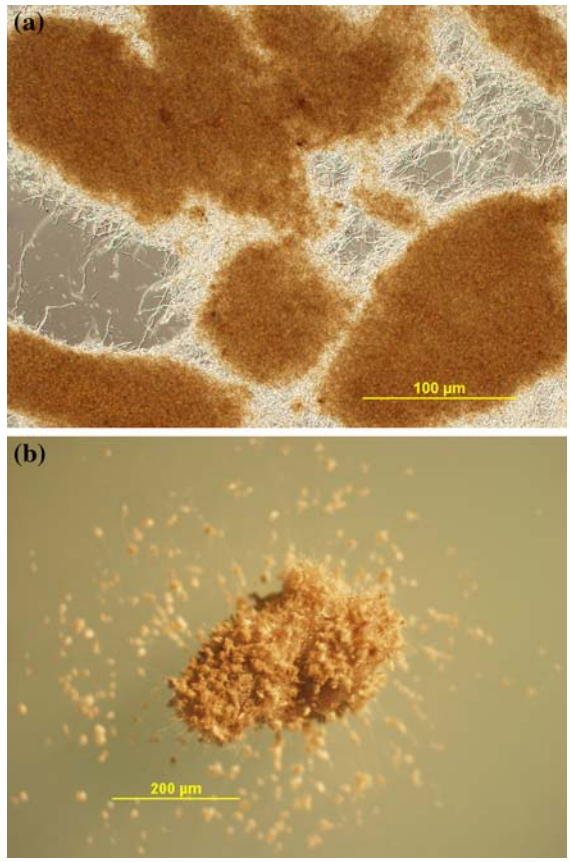

Fig. 1 Photomicrographs of melanized microsclerotia of *Metarhizium anisopliae* produced in liquid culture fermentation (**a**) and conidia production by air-dried microsclerotia-containing granules of *Metarhizium anisopliae* on water agar after incubation for seven days at 28°C (**b**). Microsclerotial granule (**b**) is covered with olive-green conidial masses and hyphal extensions from the granule are producing additional conidial masses. Photomicrographs taken with an Olympus DP70 photosystem, automatic scale calibration, on an Olympus BX60 light microscope with Nomarski optics (**a**) and an Olympus SZH10 stereo microscope (**b**)

The formation of sclerotial propagules by *M. anisopliae* in liquid culture was unexpected but is likely related to its soil-inhabiting nature (Klingen et al. 2002; Zimmermann 2007). Reports pertaining to the environmental association of *M. anisopliae* with various soil types and not to insect host suggested that the persistence of this fungus in these soils was likely unrelated to the presence of an insect host (Bidochka et al. 2001; Quesada-Moraga et al. 2007). Furthermore, the association of *M. anisopliae* with plant roots and root exudates supports the idea

that these fungi may be capable of survival in soils without an insect host (Hu and St. Leger 2002; Bruck 2005).

The ability of *M. anisopliae* to form an overwintering propagule, such as a sclerotium, would certainly provide this fungus an ecological advantage. It has been assumed that conidia were the overwintering propagule for *M. anisopliae*. This assumption is confounded by the fact that microsclerotia produce conidia when rehydrated under environmental conditions conducive to growth. This is particularly true given the fact that most studies concerning the presence of *M. anisopliae* in soil have been conducted by baiting with susceptible insects or by serial soil dilution plating onto *Metarhizium*-selective media to identify colony forming units of *M. anisopliae* (Hu and St. Leger 2002; Klingen et al. 2002; Keller et al. 2003; Meyling and Eilenberg 2006; Quesada-Moraga et al. 2007). Microscopic studies of soil are needed to determine the presence or absence of microsclerotial propagules of *M. anisopliae* and, if present, their ability to produce conidia by sporogenic germination in soil, root exudates, or decaying insect cadavers.

The liquid culture production, desiccation tolerance, and sporogenic germination of microsclerotia of *M. anisopliae* supports their use for control of soil-dwelling insects. The ability of *M. anisopliae* to produce sclerotial bodies may also provide insight into the soil-dwelling nature of this fungus. As with other fungi that produce sclerotia under the controlled conditions inherent to liquid culturing, a model is now available for discerning the processes involved in the differentiation of *M. anisopliae* hyphae to produce sclerotia under gnotobiotic conditions. Understanding and developing this biocontrol approach for soil-dwelling insects should provide microsclerotial preparations of *M. anisopliae* with distinct advantages over the use of spore- or mycelium-based insect biocontrol products.

Formulation of fungal propagules—considerations

Shelf life—environment during storage

The formulation of aerial conidia or other fungal propagules is a somewhat different paradigm than formulation of a chemical active ingredient. First of all, the spore, be it a conidium or a blastospore, must be kept alive until used. For commercial use, a mycoinsecticide must have an "acceptable shelf life" generally considered a minimal loss in spore viability for at least one year at room temperature. A typical conidial viability trend for a commercial *B. bassiana* (isolate GHA, Laverlam International, Butte, MT) is depicted in Fig. 2, where longevity is inversely proportional to temperature. Figure 3 shows the relationship of conidial half-life to storage temperature for a typical lot of commercial *B. bassiana* GHA unformulated technical powder. Storage temperatures above 30°C resulted in commercially unacceptable shelf life (<1 year) while temperatures <20°C allowed multi year storage.

A basic premise regarding the storage stability of a fungal mycopesticides is that shortened shelf life is

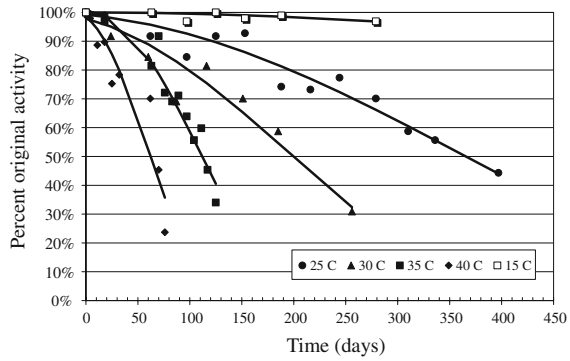

Fig. 2 Viability of *Beauveria bassiana* strain GHA technical grade (unformulated) conidial powder (Mycotech Lot 99-05-2) stored in sealed polypropylene containers at various temperatures. Lines are fitted based on linear regressions of angular transformed data (percent original activity remaining) versus time, backtransformed to percents for use in the graph

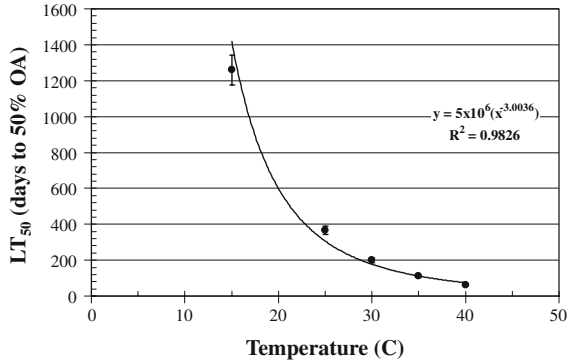

Fig. 3 Effect of storage temperature on the half-life (LT_{50} of original activity remaining, OA) of *Beauveria bassiana* unformulated, conidial powder (Mycotech Lot 99-05-2). Bars equal to one standard deviation

primarily due to spores slowly initiating germination, but dying as the succession of cues and requirements to complete germination are not fulfilled in the storage environment (Jaronski 1997). In addition to being viable, the fungal propagule must also possess the ability to infect and kill the insect host under the environmental conditions where the entomopathogen will be used. How does one keep a fungal spore alive and efficacious, yet dormant, for a satisfactory length of time? Understanding the cues that lead to spore germination and how the storage environment influences these cues is critical to the development of stable mycoinsecticidal products.

An analogy to the requirements for conidial germination is the fire prevention triangle (Anonymous 2009). Three components—fuel, oxygen, and heat, or an ignition source—are necessary for combustion. These three components can be linked conceptually to each other in a triangle. If one corner of the triangle is eliminated, fire can be prevented. Likewise, the three requirements for germination are nutrients, water, and oxygen. Eliminating one component of the germination triangle prevents spore germination. The challenge is to eliminate or inhibit a requirement for germination in the storage environment without killing or reducing the efficacy of the fungal spore.

Nutrients, the first component of the germination triangle, are very difficult to exclude from fungal spore products as they may be endogenous or in the production medium during harvest, thus becoming part of the final product. As little as 6 nM glucose can stimulate and support conidial germination in *B. bassiana* (Smith and Grula 1982). Economics preclude the harvest of mass produced fungal spores free of residual nutrients.

Water is the second component of the germination triangle. Liquid water, at least on the level of a molecular film, is necessary to convey chemical cues to the spore and to initiate germination. Excluding water, or reducing the water activity in the storage environment below a certain level, can prevent germination. This phenomenon has been reported for *B. bassiana*, *L. lecanii*, and *Metarhizium flavoviride* and is the subject of at least one US patent (Jung and Mugnier 1989; Chandler et al. 1994; Hedgecock et al. 1995; Jin et al. 1999). Of course, removing molecular water can damage fungal spores and greatly shorten their longevity (Crowe and Crowe 1986).

The third component of the germination triangle is oxygen. The complete exclusion of oxygen is difficult to achieve and may not be beneficial under some conditions. Living fungal spores continue low-levels of basal metabolic activity even under conditions adverse to growth. Measures that exclude oxygen from the storage environment e.g., vacuum-packing, inclusion of oxygen scavengers, or replacing the air in container head space with nitrogen or carbon dioxide, may be deleterious (Jaronski, unpublished data) or may have a beneficial effect to spore survival (Jin et al. 1999). Moisture levels, temperature, and nutrient availability are conditions present during storage that confound the influence of oxygen on spore survival.

Intrinsic conidial longevity under optimal storage conditions can be unique to a fungal species or even an isolate within a species (Jaronski 1997; Hong et al. 2001). In a study of six *B. bassiana* and three *M. anisopliae var. acridum* isolates from Madagascar, half-life of conidia produced under identical conditions and dried to the same endpoint of 4–6% moisture and stored at 25°C, ranged from <27 d for the *Metarhizium* isolates and >210 d for four of the *Beauveria* isolates (Jaronski 1997). In comparison, 89% of the *B. bassiana* GHA conidia germinated after storage for 266 d. More recently, in a comparison of 27 *B. bassiana* isolates and the commercial *B. bassiana* GHA isolate, the former had LT_{50}s of 19–112 days at 30°C, whereas GHA had an LT_{50} of 215 days (Jaronski, unpublished data).

The shelf life of spores of fungal entomopathogens can be affected by nutritional and environmental conditions present during production and drying. The initial moisture content of the conidial powder and the drying speed of conidia produced on solid substrate culture were shown to influence the shelf life of conidia of *M. flavoviride* and *B. bassiana* (Hedgecock et al. 1995; S. Jaronski, unpublished data). The storage stability and desiccation tolerance of liquid culture produced blastospores of *I. fumosoroseus* were influenced by the speed of drying and the form and quantity of nitrogen provided in the liquid culture medium, respectively (Jackson et al. 1997; Jackson 1999). Work by Hallsworth and Magan (1994, 1995, 1996) suggested that manipulating the polyol content within the conidia of *B. bassiana, M. anisopliae* and *I. farinosus* through nutrition and/or osmotic stress can extend the range

of water availability over which fungal propagules can germinate and may provide benefit during desiccation and storage (Hallsworth and Magan 1994, 1995, 1996).

Formulations, adjuvants, adherence, and interactions

Formulation plays an important role in delivering the fungal entomopathogen to the target environment. Formulated fungal entomopathogens are typically prepared as technical concentrates, wettable powders or oil dispersions (Faria and Wraight 2007). Technical concentrates are the fungal propagules combined with production by-products and minimal amendments. In wettable powder formulations, the dried fungal propagules are formulated to be dispersed in water and applied as a suspension. In oil dispersions, the fungal propagules are suspended in a water immiscible liquid which is intended to be diluted in water before use. Oil dispersions are typically limited to use with hydrophobic conidia. Both of these aqueous suspensions would typically be applied with spray applicators. In some cases, however, a wettable powder formulation has advantage over the liquid formulations such as when oil-incompatible materials have been applied to the crop. For example, use of an oil-based formulation on a crop treated with elemental sulphur can cause severe phytotoxicity (Hoy 2008). Recipes of wettable powder formulations must be carefully created to maintain spores and inerts in suspension with minimal agitation during spraying.

There are several important variables to consider when developing formulations which will be applied as aqueous sprays. First, aerial conidia of *Beauveria* spp., *Metarhizium* spp. and *Isaria* spp. are highly hydrophobic due to glycoproteins arranged in overlapping rodlets on the conidial surface (Bidochka et al. 1995). This property makes oil carriers ideal for these conidia. For example, *M. acridum* conidial powder is routinely suspended in groundnut oil, No. 2 diesel or kerosene for ultralow volume application against Orthoptera in Africa and maize oil in Australia. The suspension of aerial conidia in water is very difficult without the use of a wetting agent. A wetting agent must be selected that does not interfere with the infection process, much less kill the fungal propagule. Additionally, consideration should also be given to the fact that some wetting agents and adjuvants have been shown to expand the host range of fungal plant pathogens and may have a similar impact on insect pathogens (Boyette and Abbas 1994; Hoagland et al. 2007).

Secondly, spray formulations are designed to deliver the fungal entomopathogen directly to the insect (contact insecticide), to a location where protection is desired (plant surfaces, post harvest storage areas, etc.) or to areas frequented by the insects. Understanding how the spray droplet and entomopathogenic propagule interact with the target surface can help in guiding formulation decisions. In order for a spray droplet to adhere to a surface, the droplet must first be able to wet the surface. In general terms, for a liquid to wet a solid, the surface tension of the liquid must be lower than the surface energy of the solid. Most of the targets for fungal entomopathogen spray applications are hydrophobic or low surface energy targets (insect cuticles, plant surfaces, etc.). These types of surfaces are commonly referred to as being hydrophobic, since they repel water or the interaction with water is not energetically favourable. Surfaces with low energy are difficult to wet with aqueous solutions, since the surface energy (surface tension for a liquid) of water must be reduced below that of the solid surface for wetting to occur. In order to reduce the surface tension of aqueous solutions low enough to wet these surfaces, surfactants are added, which greatly lower the surface tension of water.

Surfactant selection must meet two criteria for effective use in a fungal entomopathogen formulation: biocompatibility and physical property performance. Biocompatibility is typically tested explicitly with potential surfactants, but there are general guidelines that can narrow one's search. Aerial conidia are generally much more tolerant of surfactants than blastospores, submerged conidia, or hyphal formulations and many successful examples of their use are available in the literature (Daoust et al. 1983; Alves et al. 2002; Akbar et al. 2005; Faria and Wraight 2007; Jin et al. 2008). Blastospores, submerged conidia, and hyphae lack the hydrophobic properties of aerial conidia, which allows surfactants to interact directly with the outer membrane of the cell. These hydrophilic propagules require more discretion when selecting a potential surfactant. The antifungal activity of surfactants is often correlated with lipophilicity of the surfactant (Leal et al. 2009) or more specifically the length of the alkyl chain

(Oros et al. 1999; De Jonghe et al. 2007). The longer the alkyl chain of the surfactant the more lethal they tend to be to yeasts and filamentous fungi. Alternatives with reduced fungicidal activity are available to replace the traditional alkyl chain based surfactants, including surfactants based on branched alkyl chains (Ayala-Zermeno et al. 1999), block co-polymers (Baur et al. 1997) and protein hydrolysates (Dunlap et al. 2007). These surfactants all limit the length of alkyl chains that could enter the membrane, which reduces their toxicity to the fungus. Nevertheless, the effect of wetting agents needs to be empirically determined as compatibility will differ among fungal species (Jaronski 1997). There is potential for differences between isolates in sensitivity to a particular chemical. In some cases, there is a concentration dependent effect of the emulsifier or dispersant on shelf life. Lastly, chemical interactions among formulation ingredients can have an effect on the conidia. In one example, the addition of an inert ingredient into a wettable powder formulation countered the deleterious effect of a dispersant on the shelf life of a commercial *B. bassiana* at 30°C and 35°C, although the dispersant had no effect on these conidia at 5–25°C (Jaronski 1997).

Physical property performance in the selection of a surfactant is often guided by the ability of the surfactant to reduce the surface tension of aqueous solutions. In addition to the ability to lower the equilibrium surface tension of water, an important parameter in surfactant selection is the dynamic surface tension. Dynamic surface tension is important in spray applications because during the spraying/droplet-forming process, new droplet surfaces are constantly being created and the surfactant must diffuse to the surface to reduce the surface tension. The time window between the droplet leaving the sprayer and hitting the target is often very short. If the surfactant has not sufficiently diffused to the surface of the droplet before impact, the surface tension will not be lowered and the surfactant will have provided little to no benefit. A universal spray droplet adhesion model has been proposed for the leaf surfaces (Forster et al. 2005) and its concepts are extendable to other surfaces (i.e., insect cuticle).

Understanding how fungal entomopathogen propagules interact with their insect host or respond to their target environment are important considerations when developing formulations. The target environment may be the insect cuticle or the physical environment in which the propagules are applied, such as, the phyllosphere, rhizosphere, insect nest, etc. There are distinct sequential events required for successful infection: initial attachment through non-specific interactions, adhesion through specific or induced interactions, conidial germination (which has several phases), chemotaxis of the hyphal tip on the cuticle, appressorium formation, and penetration into the cuticle (St. Leger 1991). During the infection process, fungal entomopathogenic propagules interact with their environment (the insect cuticle) through specific and non-specific interactions. Non-specific interactions mediate the initial contact of the propagule with surfaces. Such interactions arise from the physicochemical properties of the propagule surface and include hydrophobic, polar, and electrostatic properties. Specific interactions occur during germination and penetration and are directed responses of the fungus to specific cues on and in the cuticle. Formulation considerations are usually limited to the non-specific interactions. Knowledge of the surface physicochemical properties provides a basis for predicting how these propagules will interact with their insect hosts and their hosts' environment. These physicochemical properties have been reported for three entomopathogens, *I. fumosorosea*, *N. rileyi*, and *B. bassiana* (Pendland et al. 1994; Dunlap et al. 2005; Holder et al. 2007).

The interactions between a microbe and a surface can be described under defined conditions (e.g., pH and ionic strength) using Derjaguin–Landau–Verwey–Overbeek (DLVO) theory (Derjaugin and Landau 1941; Verwey and Overbeek 1948), extended DLVO theory or a thermodynamic approach (van Oss 1995). This information can be the basis for understanding interactions with formulation adjuvants and for choosing formulation conditions which improve adhesion. It will also be useful in predicting the transport properties of propagules, once applied to the host environment, such as transport in soils (Horn et al. 2001), mulches (Sun et al. 2008) or the phyllosphere (Bora et al. 1994). If the surface energy of the target surface (insect cuticle, soil, etc.) is known, propagule-surface interactions can be quantified and possibly optimized through growth conditions (Jana et al. 2000; Shah et al. 2007) or formulation (Webb et al. 1999). Specific interactions typically occur after the initial adhesion of conidia to

the insect cuticle. Relatively little is known about these specific interactions. In *M. anisopliae*, the presence of a protein, MAD1, mediates the adhesion of conidia to insect cuticle while the MAD2 protein mediates adhesion to plant cuticle (Wang and St. Leger 2007). The use of microarrays to determine which genes are activated during the infection process should lead to a clearer understanding of these specific interactions (Wang and St. Leger 2007).

Persistence in the insects habitat

Secondary acquisition of infective propagules by insects from sprayed plant surfaces is often equally or more important than direct propagule contact from the spray (Fernandez et al. 2001; van der Valk 2007). The persistence of fungal propagules in the environment can be a very important factor in the overall efficacy of a mycoinsecticide, yet the persistence of propagules of fungal entomopathogens in the environment is generally poor. Estimates of persistence in field situations with UV exposure vary from a few hours to a few days with an exponential decay relationship (Inglis et al. 1993: McCoy et al. 2002; van der Valk 2007). Recently, a number of materials have been found to have value in improving persistence (Leland et al. 2004; Leland and Behle 2005; Reddy et al. 2008; Villamizar et al. 2009). The most promising technology to date is an organo-clay matrix containing one of several food grade dyes (Cohen and Tammar 2009)

Another factor affecting fungal propagule persistence is rain. When applied to leaf surfaces as an aqueous suspension, conidia readily washed off with simulated rain (Inglis et al. 2000). Conidia in an oil formulation, however, greatly reduced conidia loss from the leaf surface; oil-in-water emulsions were intermediate in effect. The use of polymeric stickers and spreaders may increase rainfastness but be counter productive if they prevent the transfer of conidia from the contacted surface to the insect cuticle.

Insect behaviour-based mycoinsecticide delivery

Formulation can be used to improve the delivery of fungal entomopathogens to their host or host environment. Many insect pests reside in difficult-to-reach locations, such as on the undersides of leaves, inside branches, stems or fruit, or in underground nests. While the application of liquid formulations is limited by gravity, foams can expand and deliver fungal propagules to hard-to-reach areas. For example, a foam formulation was developed to deliver blastospores of *I. fumosorosea* to termite nests located in trees or building structures (Fig. 4; Dunlap et al. 2007).

Fig. 4 Application of a biocompatible foam formulation of *Isaria fumosorosea* blastospores (**a**) through a hole drilled in the base of a Formosan termite-infested tree. Blastospores of *I. fumosorosea* are carried upward through termite galleries within the tree, emerging from pre-drilled holes above the injection site (**b**), for improved contact, infection, and control of Formosan subterranean termites

Another potential area to exploit in formulation development is the blocking or interfering with the insect's ability to detect the fungal entomopathogen within its body or to initiate a defence response. Insect defences are based on the innate immune system of the insect and consist of humoral and cellular responses (Lavine and Strand 2002). Insects have evolved receptors that bind to conserved

molecules presented by microbial pathogens to identify the specific pathogen attacking them (Fearon 1997). These receptor- based systems identify the specific pathogen through recognition of specific pathogen-associated molecular pattern motifs. The detected molecular motifs are usually cell wall components of the pathogen such as, lipopolysaccharides, peptidoglycans and β (1,3)-D-glucans (Wang and Ligoxygakis 2006; Müller et al. 2008). This strategy was recently used to improve the virulence of *M. anisopliae* against termites. Bulmer et al. (2009) demonstrated that termites exhibit a unique β (1, 3)-glucanase activity in their tissues, cuticular washes, and nest material. The β (1, 3)-glucanase activity was proposed to have two functions related to termite defence. It acts as an environmental sensor by cleaving and releasing pathogen components, which activate the termite defence systems. The second function is to cleave and weaken the pathogen cell wall, making the pathogen more susceptible to the termites' antimicrobial peptides. By combining *M. anisopliae* with a β (1, 3)-glucanase inhibitor, improved biocontrol efficacy was demonstrated (Bulmer et al. 2009). It is easy to envision formulations of fungal entomopathogens combined with small molecules that inhibit insect defence and recognition pathways.

Formulation technology also plays a role in "bait and kill" or "lure and kill" applications as summarized by Vega et al. (2007) and Baverstock et al. (2009). These applications exist in a wide variety of formats. The bait/lure mechanism takes advantage of innate insect behaviour in response to various cues. The use of environmental stimuli such as color (Avery et al. 2008) and preferred habitat, ex. clay water pots to attract mosquitoes (Farenhorst et al. 2008), have been used to attract insects for the dissemination of fungal entomopathogen propagules. Semiochemicals have also been used to lure various insects to a fungal entomopathogen infection site (Vega et al. 2007). Insects targeted using this biocontrol approach include ticks [*Amblyomma vaiegatum* (Maranga et al. 2006; Nchu et al. 2009)], aphids [*Phorodon humuli* (Hartfield et al. 2001)], Japanese beetle [*Popilla japonica* (Klein and Lacey 1999)], grain borers [*Prostephanus truncatus* (Smith et al. 1999)], and the diamond-back moth [*Plutella xylostella* (Furlong et al. 1995)].

Autodissemination devices based on a commercial, pheromone-based Japanese beetle trap are currently being deployed on an operational basis in the Azores (S. Jaronski, unpublished data). Food attractants have been combined with fungal entomopathogens and have had some success controlling a variety of insects, such as termites [*Reticulitermes flavipes* (Wang and Powell 2004)], ants [*Atta cephalotes* (Lopez and Orduz 2003)], locusts [*Schistocerca gregaria* (Caudwell and Gatehouse 1996)] and house flies [*Musca domestica* L. (Renn et al. 1999)]. Vegetable oils rich in oleic, linoleic and linolenic acids, that stimulate necrophagy among grasshoppers (Orthoptera: Acrididae), have been used to attract the insects to a toxicant or to an infective fungal propagule (Bomar and Lockwood 1994a, b; Latchininsky et al. 2007). Linoleic and linolenic acids stimulate necrophagy in many species of grasshoppers and an oil carrier, rich in these compounds, can serve as the basis of a mycoinsecticide attracticide. These attracticides are useful in strip treatments thus reducing the overall rate of mycopesticide application per protected area (Lockwood et al. 2001). Canola olive and flax oils are rich in these compounds. Canola oil has been used to validate the principle in the field with carbaryl (Bomar and Lockwood 1994b). These findings are being extended to mycoacaricide use on US rangeland (Jaronski et al. unpublished).

Formulations may also be used to reduce the repellence of fungal materials or reduce the stimulation of behavioural responses by insects. For example, the conidia of fungal entomopathogens stimulated diverse defensive behaviours in termites that served to eliminate or minimize the impact of the pathogen on the colony (Rosengaus et al. 1998; Fefferman et al. 2007). These nest hygiene behaviours include intense grooming of workers, disposal or isolation of infected workers and sporulating cadavers, etc. Blastospores of *I. fumosorosea* were shown to be much less repellent to the Formosan subterranean termites *Coptotermes formosanus* when compared to solid substrate produced conidia of *I. fumosorosea* (Wright et al. 2003). In addition, vegetative mycelium has been shown to be readily accepted by termites and taken into termite nests where it sporulates (Stamets 2006). Evidently, presporogenic mycelium of *Metarhizium* spp., *Beauveria* spp. or *Cordyceps* spp. contain attractant volatiles that induce social insects such as ants and termites to graze on the mycelium, scattering the mycelium around feeding areas and nesting chambers,

after which the fungus sporulates to produce infectious conidia (Stamets 2006). This presporogenic mycelium can be introduced to termites or ants on grain-based solid substrate or freeze-dried mycelium.

These "lure and kill" strategies ultimately rely on the transfer the infective propagule of an entomopathogenic fungus to the insect when it arrives at the dispenser. In this strategy, the dispenser can be any device the insect is lured to which contains the fungal entomopathogen propagules. Little is known about optimizing parameters to improve the transfer of viable fungal propagules to the insect. A basic understanding of the physical and environmental parameters that impact fungal propagule transfer to the insect cuticle would benefit the use of this strategy

Conclusion

The control of insect pests with fungal entomopathogenic fungi requires a significant basic understanding of the interactions between the target insect, fungal entomopathogen, and environment. The production and formulation goals for these fungal entomopathogens must consider economic realities but also be mindful of the ecological constraints and requirements for consistent insect infection and control. An understanding of the critical biotic and abiotic constraints for each particular insect pest–environment will guide the selection and development of these agents. New approaches to the production of efficacious fungal propagules and the development of formulations tailored to overcome specific environmental constraints will lead to the availability of dependable entomopathogen-based bioinsecticides for controlling insects in a wide variety of habitats.

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